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(54) Title: MONOCLONAL ANTIBODY

(57) Abstract

A monoclonal antibody having a greater binding efficiency to D sub-type human interferon-α than to A sub-type human interferon-α. A particular monoclonal antibody is designated YOK5/19. A process is described for the preparation of the monoclonal antibody and uses of the monoclonal antibody in immunopurification and immunoassay are described.

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MONOCLONAL ANTIBODY

FIELD OF THE INVENTION

This invention relates to a monoclonal antibody. In particular the invention relates to a monoclonal antibody to human interferon -α, a process for the preparation of the monoclonal antibody, the use of the monoclonal antibody in an immunoassay for human interferon -α, the use of the monoclonal antibody in a process for the immunopurification of a sample containing human interferon -α and an assay for antibody to human interferon -α.

BACKGROUND OF THE INVENTION

Interferon is the generic name for a group of proteins which exhibit such properties as antiviral activity and cell growth inhibition (Stewart W.E. (1979). "The interferon system". Springer, Vienna and "The Biology of the interferon system." Elsevier-North Holland Biochemical Press, Amsterdam). The interferons may be divided into three main types; interferon-α (IFN -α or leukocyte interferon), interferon-β (IFN-β or fibroblast interferon) and interferon -γ (IFN-γ or immune-interferon) (Stewart W.E. et al (1980) "Interferon Nomenclature", Nature, London 286. 110). The present invention relates to a monoclonal antibody to interferon-α and in particular to human interferon-α (Hu-IFN-α).

In published co-pending British patent application GB 2083836A (also published as international published application WO 81/02899) there is described a monoclonal antibody to Hu-IFN-α. The monoclonal antibody is secreted by a cell line prepared by the cell fusion process of Kohler and Milstein (Kohler and Milstein, Nature 256, 495-497). A particular embodiment of the invention

claimed in GB 2083836A is a monoclonal antibody secreted by a cell line denoted NK2. The co-pending application also describes an immuno-purification process using monoclonal antibody from NK2.

It has now been discovered that monoclonal antibody to human interferon-α, as derived from the cell line NK2, does not completely remove the interferon activity of an interferon-containing sample passed through an immuno adsorbent column comprising the antibody immobilised upon a solid phase. This results in a fractionation of such a sample which may in some circumstances be undesirable. The broad effect has been noted by a number of workers (Meurs E. et al Infection and Immunity (1982) 37 No.3 pp 919-926; Staehelin T. et al Proc. Natl. Acad. Sci. (1981) 78 No.3 pp 1848-1852; Allen G et al J. Gen. Virol 63 pp 207-212). The results are said to suggest that monoclonal antibody to human interferon-α does not bind all the components of a given sample and it is further suggested that in order to use monoclonal antibody affinity chromatography for the purification of the complete spectrum of interferon-α components, it will be necessary either to find another antibody which binds all components, or to use a combination of two or more antibodies with complementary specificities.

It is the object of the present invention to provide a monoclonal antibody having a complementary specificity to that derived from the NK2 cell line.

Hu-IFN-α comprises a number of distinct molecular entities, known as sub-types. There are eight to twelve identified sub-types of Hu-IFN-α the relative proportions of which are yet to be established. The sub types are each about 165 amino

acid residues in length and have many homologous features. It is thought that in all there may be up to 20 different sub-types of natural Hu-IFN-α since 20 different genes for Hu-IFN-α have to date been recognised. For historical reasons, the nomenclature of the Hu-IFN-α sub-types is not standard, one method relying upon an alphabetical system and an alternative method relying on a numerical system. The relationship between them is as follows:

A	B	C	D	E	F	G	H	I	J	K
α2	α8	α10	α1	α4	α3	α5	α6	α7		

The alphabetical nomenclature will generally be used in this specification.

I have discovered that the monoclonal antibody derived from the NK2 cell line binds preferentially to A sub-type human interferon (Hu-IFN- α2). Surprisingly I have also found that monoclonal antibodies which bind preferentially to D sub-type human interferon (Hu- IFN- α1) than to A sub-type human interferon act very efficiently as complementary antibodies to that derived from the NK2 cell line in the practice of monoclonal antibody affinity chromatography.

SUMMARY OF THE INVENTION

According to a first aspect of the present invention there is provided a monoclonal antibody to human interferon-α wherein the monoclonal antibody has a greater binding efficiency to D sub-type human interferon-α than to A sub-type human interferon-α. Preferably the monoclonal antibody has a greater binding efficiency to D sub-type human interferon- than to any of the other sub-types of human interferon-α. Preferably the monoclonal antibody is produced by the cell line designated YOK5/19.

The term "binding efficiency" as used herein is a measure of the relative affinity of a monoclonal

antibody for a particular sub-type of human interferon-α. The binding efficiency not only reflects the specificity of a monoclonal antibody but also the avidity of the immunochemical bond formed between the monoclonal antibody and its corresponding antigenic determinant.

The term "complementary binding efficiency" as used herein, refers to a binding efficiency which complements that of the monoclonal antibody according to the first aspect of the invention. An example of an antibody having a complementary binding efficiency is a monoclonal antibody derived from the NK2 cell line.

The NK2 cell line and its preparation are described in detail in published British patent application GB 2083836A (see also international published application WO 80/02899).

The binding efficiency of a monoclonal antibody to a human interferon-α sub-type is preferably measured by immobilising a sample of the sub-type on a solid support (for example by way of an immobilised monoclonal antibody to human interferon-α). A radioactively labelled monoclonal antibody under test is then incubated with the immobilised pure sub-type and after rinsing, the specific radioactivity of the solid support is measured. After making a numerical allowance for a background level of radiation due to non-specific binding of the labelled monoclonal antibody, the bound radioactivity (in counts per minute referred to hereinafter as cpm) is a measure of the binding efficiency of the monoclonal antibody to the sub-type. In an alternative preferred technique for measuring the binding efficiency of a monoclonal antibody to a specific sub-type of human interferon-α, a neutralization test may be employed

in which for example the binding efficiency is assessed by the measurement of the inhibition of viral RNA synthesis which results from unneutralized interferon remaining in a test sample following admixture of a sample of the sub-type of human interferon-α and the monoclonal antibody.

According to a second aspect of the present invention we provide a composition comprising, in combination, a monoclonal antibody according to the first aspect of the present invention and a monoclonal antibody of complementary binding efficiency. Preferably the monoclonal antibody of complementary binding efficiency is derived from the NK2 cell line.

The advantage of such a composition is that the two monoclonal antibodies of complementary binding efficiency have in combination a high binding efficiency for most human interferon-α sub-types.

According to a third aspect of the present invention we provide a process for the immunopurification of a sample containing human interferon-α in which either a monoclonal antibody according to the first aspect of the present invention or a composition according to the second aspect of the present invention is immobilised upon a solid support form an immunopurification medium and the sample containing human interferon-α is contacted with the medium. The antibody may for example be immobilised upon a particulate solid support. Each particle of the support may have attached a monoclonal antibody of the first aspect of the invention, an antibody of complementary binding efficiency or both. A mixture of particles may be used to produce an immunopurification column.

According to a fourth aspect of the present invention we provide a process for the immuno-

purification of a sample containing human interferon-α wherein the sample is passed sequentially, in either order, through an immunopurification column comprising, immobilised, a monoclonal antibody according to the first aspect of the present invention and an immunopurification column comprising, immobilised, a monoclonal antibody of complementary binding efficiency. Preferably the monoclonal antibody of complementary binding efficiency is dereived from the NK2 cell line. The columns may be separate or may be integral.

According to a fifth aspect of the present invention we provide an immunoassay for human interferon-α comprising the use of an antibody according to the first aspect of the present invention.

Preferably the assay uses

a sample to be assayed for human interferon-α ,

a first antibody to human interferon-α , the first antibody being bound to a solid phase support and

a second antibody to human interferon-α , the second antibody having a label attached thereto,

wherein one of the first and second antibodies is a monoclonal antibody according to the first aspect of the present invention and the other antibody is either a polyclonal antibody to human inteferon-α or a monoclonal antibody of complementary binding efficiency

the assay comprising the steps of

placing the sample, the first antibody and the second antibody in contact, in any order or combination, and

measuring the amount of second antibody bound to the solid phase through human interferon-α and the first antibody.

Preferably the sample is placed in contact with the first antibody in one step, followed by the addition of second antibody. Preferably the first antibody is a polyclonal antibody (for example sheep anti interferon-α) and the second antibody is a monoclonal antibody according to the first aspect of the present invention. Preferably the label is a radioactive label but may be for example an enzyme, a chromophore, a fluorophore, a chemiluminescent chemical group or any other moiety capable of producing a detectable signal. Preferably the polyclonal antibody to human interferon-α is sheep anti-interferon-α. Preferably the monoclonal antibody of complementary binding efficiency is derived from the NK2 cell line.

According to a sixth aspect of the present invention we provide an immunoassay for a first antibody to human interferon-α in which is used

a sample to be assayed for the first antibody to human interferon-α

a solid phase support having bound thereto a second antibody to human interferon-α, the second antibody being bound to human interferon-α

a third antibody, the third antibody having a label attached thereto and being capable of binding to the first antibody,

the assay comprising the steps of

placing the solid phase support in contact with the sample, thereby allowing first antibody to bind to the human interferon-α attached to the solid phase through the second antibody,

placing the solid phase support in contact with the third antibody thereby allowing the third antibody to bind to any first antibody which is attached to the solid phase through the human interferon-α and the second antibody, and

measuring the amount of third antibody associated with the solid phase. Preferably the second antibody is sheep anti-interferon and the third antibody is radioactively labelled sheep antibody to the first antibody. When using the assay for an antibody according to the first aspect of the present invention the second antibody may be an antibody having a complementary binding efficiency and is preferably a monoclonal antibody to human interferon-α derived from the NK2 cell line.

According to a seventh aspect of the present invention we provide a process for the production of a hybridoma cell line capable of secreting a monoclonal antibody according to the first aspect of the present invention comprising the steps of

immunizing an animal with human interferon-α,

allowing the immune system of the animal to generate lymphocytes to human interferon-α,

preparing a sample of spleen cells taken from the animal

fusing the spleen cells with myeloma cells to form a colony of hybridoma cells, and

screening the colony of hybridoma cells for cells secreting monoclonal antibody according to the first aspect of the invention

wherein the screening step employs an assay according to the sixth aspect of the invention, the third antibody having been passed through an immunopurification column, comprising immobilised antibody of a complementary binding efficiency, prior to use in the assay. Preferably the antibody of complementary binding efficiency is a monoclonal antibody derived from the NK2 cell line. Preferably the third antibody is radioactively labelled sheep antibody to the first antibody. Preferably the second antibody is a monoclonal antibody derived from the NK2 cell line.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the results of an anti-interferon binding assay for a series of dilutions of a sample containing YOK5/19 antibody

Figure 2 is a bar chart showing the results of an IRMA assay conducted on a panel of interferons using two monoclonal antibodies

Figure 3 is a bar chart showing the results of anti-interferon binding assay on a panel of interferons using YOK5/19

Figure 4 is a bar chart showing the results of an IRMA assay conducted on a panel of interferons using a monoclonal and a polyclonal antibody.

DETAILED DESCRIPTION OF EMBODIMENTS

Monoclonal Antibody Preparation

The monoclonal antibody was prepared by immunising a rat with human interferon-α, fusing rat spleen cells with myeloma cells, and selecting clones secreting the desired type of antibody.

The antigen used in the immunisation was human interferon-α prepared from leucocytes (Hu-IFN-α (Le)) ("P-IF") (Cantell et al., 1981) and was obtained from Dr. K. Cantell, Helsinki (Batch 191207 8-A, 38 x 10^6 U/ml, 28 mg/ml total protein). The antigen was diluted in phosphate buffered saline (PBS) to 2 x 10^6 U/ml, divided into 0.5 ml aliquots and stored at -20°.

At approximately weekly intervals (Table 1) an aliquot was thawed and injected without adjuvant into multiple sub-cutaneous sites on the neck and back of a young adult rate of strain LOU. Before each immunisation a blood sample was taken from the tail. After about 19 months the rate was boosted by injecting 2 x 10^6 U Hu-IFN-α that had been produced in leucocytes and purified by immunoadsorption chromatography on NK2-Sepharose 4B columns

("NK2-IFN"). This interferon was emulsified in Incomplete Freund's Adjuvant and injected intra-muscularly and sub-cutaneously. A final boost was given about three weeks later by intra-venous (tail vein) injection of interferon (P-IF) in PBS (2 x 106 U).

TABLE 1: Immunisation Schedule

Day	Injection
1	106U P-IF
7	" "
14	" "
23	" "
29	" "
35	" "
42	" "
49	" "
576	2 x 106U NK2-IF + adjuvant
599	2 x 106U P-IF
604	(Animal killed)

To follow the progress of the immunisations the rat was bled from the tail and serum samples collected at approximately weekly intervals and the sera tested for their ability to neutralise the antiviral activity of IFN-α in a plaque reduction assay. In this assay 10U of IFN, when incubated on a monolayer of normal human cells before the addition of a titred dose of vesicular stomatitis virus, resulted in a reduction in the number of viral plaques to about 20% of the number of plaques in the control (no IFN). Preincubation of the interferon with a sample of the rate serum (at a final dilution of 1/60) before adding to the cell monolayer, gave in some cases restoration of the number of plaques (see Table 2).

On day 604 the rat was filled and the spleen removed aseptically. Using standard procedures (see Galfre & Milstein, 1981), 108 spleen cells were fused to 6 x 107 cells of the non-secreting variant derived from a rat hybridoma line (YB2/3.0 Ag.20) (Galfre, Milstein & Wright, 1979). The remaining spleen cells were cryopreserved in liquid nitrogen.

Following fusion the cells were dispersed into 48 2 ml cultures in 24 well plates (Linbro). Three weeks after fusion hybrid colonies were observed in 23/48 wells.

TABLE 2:

Day of Bleed	Neutralisation of IFN
0	-
34	+
41	+
48	+++
55	+++

Where - = no effect of serum
+ = some restoration of plaque number
+++ = 80% of restoration of plaque number.

Three weeks after fusion the culture supernatants were tested in a assay designed to detect anti-interferon antibodies. This assay is a modification of that designed to measure interferon concentrations by immunoradiometric assay (Secher, 1981). sheep anti-interferon antibodies are coated onto a plastic substratum. This may be in the form of tubes or beads or the wells of microtiter trays. We preferred the us of 96-well microtiter trays. 100 µl of sheep anti-interferon (purified IgG

fraction; 10-25 µg/ml in PBS/0.1% NaN_3, 5 mM ethylene diamine tetra-acetic acid) is added per well and incubated at 4°C for 16 h. The antibody solution is then removed and the wells filled with "Blocking Medium" (PBS containing 1% (v/v) normal human plasma, 0.5% bovine serum albumin (BSA), 0.1% NaN_3) and incubated for at least 1 hour at room temperature (or overnight at 4°C). After removal of the blocking medium the wells were washed twice with PBS, 0.5% BSA, 0.1% NaN_3. A solution containing Hu-IFN-α (100 µl, about 4000 V/ml in Blocking Medium) is added to each well except for control wells, to which 100 µl Blocking Medium is added. After a 2 hour incubation at 20-25°, the interferon is removed and 100 µl of the solution to be tested is added to the well. (Typically this would be cell culture supernatant). After a further incubation (about 2 hrs at 20-25°) the test solution is removed and the wells washed twice with PBS, 0.5% BSA, 0.1% NaN_3. Care should be taken to remove all the test solution and to perform the washes as quickly as possible.

The next step involves incubation with labelled anti-rat Ig antibody. 100 µl of ^{125}I-labelled sheep anti-rat immunoglobulin antibody, (5×10^5 - 10^6 cpm/ml, 1 µCi/µg, affinity purified sheep antibody in PBS, 0.5% BSA, 0.1% NaN_3) is added to each well and incubated for about 2 hours at 20-25°. The unbound labelled antibody is then removed and the wells washed twice more with PBS, 0.5% BSA, 0.1% NaN_3.

The radioactivity remaining in each well is a measure of the bound sheep anti-rat Ig, which in turn is a measure of the rat anti-interferon antibody that bound to the solid-phase via the interferon-sheep anti-interferon bridge. This radioactivity may be measured by cutting off the bottom of the wells with a hot wire and transferring each well to a tube for

counting in a gamma counter.

An alternative form of this assay uses monoclonal antibody (e.g. NK2) attached to the plastic instead of sheep anti-interferon. In this case the labelled sheep anti-rat Ig antibody should be passed through an NK2-Sepharose column before use to remove any antibodies that bind to NK2. This form of the assay has the advantage that only antibodies recognising a distinct antigenic determinant from that recognized by NK2 will give a positive signal, but antibodies that bind only to IFN species that lack the NK2 determinant will not lead to a positive signal.

Using the alternative form of the assay (NK2-coated plastic) the supernatants from all the cultures containing actively growing cells were tested three weeks after the fusion for the presence of anti-interferon antibodies. The results (Table 3) suggested that there was a single culture (YOK5/19) producing anti-interferon antibodies that can bind to IFN-α at the same time as NK2 antibody. Since the IFN-α molecules are monomeric this suggests that the YOK5/19 antibody and the NK2 antibodies recognise distinct antigenic sites. The assay was repeated on culture supernatants taken 4 weeks and 5 weeks after the fusion, and the activity remained stable.

TABLE 3:

Sample	CPM BOUND x 10-2	
	+ IFN	- IFN
Serum from immunised rat (1/1000)	61	8
YOK5/19 culture SN	16	6
21 other culture SN	5-9	5-8

Cloning and Production of Monoclonal Antibody

Cells were serially diluted and at each dilution plated out into Microtiter trays (96-wells x 0.2 mls). Cell culture supernatants were taken at 10-14 days from the trays seeded at the highest dilution at which cell growth was still observed and tested in the assay. Cells from the most strongly positive culture (YOK5/19 (3)) were grown up, frozen in liquid nitrogen for long-term storage and about 5 x 107 cells injected into the peritoneal cavity of F1 AO x LOU rats that had previously been primed with "pristane" (Galfre & Milstein, 1981). Ascitic tumours developed and after about two weeks cells were harvested from the peritoneum and returned to culture. The cells continued to grow well and produce anti-interferon antibody. These cells (YOK5/19 (3) (As)) were again subjected to dilution fractionation as above and a culture, YOK5/19 (3) (As) (3.80) selected on the basis of a binding assay (Table 4) using sheep anti-interferon and either crude Hu-IFN-α (Le) or a cloned IFnα1 . Cells from this culture were then successfully cloned on semi-solid support as described (Galfre & Milstein, 1981) except that agarose was used instead of agar and mouse peritoneal cells were attached to the petri dishes before addition of the agarose. This was achieved by rinsing out the peritoneal cavity of a freshly killed Balb/C mouse with cell culture medium (2 x 8 ml) from a hypodermic syringe. The cell suspension was then divided amongst 10-20 9 cm petri dishes and incubated for 4-24 hours. After this incubation the culture medium was removed and the molten agarose (0.5% in medium) added.

TABLE 4:

Sample	cpm bound (x 10-2)	
	Hu-IFN-α (Le) (Le)	Hu-IFN-α1
YOK5/19	7	8
YOK5/19 (3)(As)(3.50)	8	8
YOK5/19 (3)(As)(3.53)	0	0
YOK5/19 (3)(As)(3.59)	7	7
YOK5/19 (3)(As)(3.65)	4	6
YOK5/19 (3)(As)(3.76)	8	6
YOK5/19 (3)(As)(3.78)	7	7
YOK5/19 (3)(As)(3.79)	0	0
YOK5/19 (3)(As)(3.80)	8	6
YOK5/19 (3)(As)(3.80) 1/10 dilution	7	8

* after subtraction of non-specific binding (100-200 cpm)

Clones were picked from a petri dish containing about 20 clones, and 10 out of 13 tested were positive in the anti-interferon assay.

A recloning of the selected clone YCK5/19(3)(As)(3.80).31 was carried out and the clone YOK5/19(3)(As)(3.80).31.9 isolated. This clone was renamed YCK5/19.31.9 and the antibody produced by it called simply YOK5/19 antibody.

To obtain serum and ascites fluid containing YOK5/19 antibody cells from culture (1-5 x 107 per rat) were injected intra peritoneum or subcutaneously into F1 hybrid rats of the AO x LOU strains as described (Galfre & Milstein, 1981). Cells of the following clones were used with no significant difference observed: YOK5/19(3)(As)(3.80).8
YCK5/19(3)(As)(3.80).22
YOK5/19(3)(As)(3.80).31
YCK5/19(3)(As)(3.80).31.9.

10-20 days after injection the rats were killed and the ascites fluid and/or blood removed. Blood was allowed to clot and the serum removed and stored at -20°. Ascites fluid was centrifuged to separate the cells, and the supernatant was removed and stored at -20°. Cells from the ascites fluid or from a suspension prepared from a solid subcutaneous tumour were injected into fresh rats to passage the cell line. Serum and ascites fluid samples were subjected to cellulose acetate electrophoresis (Microzone, Beckman) to monitor the production of YOK5/19 antibody in the animal,

To purify YCK5/19 antibody the following protocol was used:

To 100 ml pooled serum ascites fluid 100 mls saturated ammonium sulphate solution was added at 4°C with stirring. The precipitate was collected by centrifugation at 10,000 rpm (MSE21) x 20 mins and redissolved in 10mM sodium phosphate buffer, pH7.5 (44 mls). This solution was dialysed against 10 mM sodium phosphate pH7.5 (5 x 2 litres) and, after

centrifugation (10,000 rpm x 20 mins) to remove any denatured, insoluble protein, loaded onto a column of DE52 (7.5 cm x 3.85 cm, Whatman) equilibrated in the same buffer. The column was then eluted with 420 ml of 10 mM sodium phosphate buffer, pH7.4, followed by a linear gradient of 10-100 mM sodium phosphate, pH7.4 (800 mls + 800 mls). Fractions were collected (12.3 mls) and the absorbance (280nm) of the column eluate continuously monitored. The fractions comprising the first peak to elute after the beginning of the gradient were identified as pure YOK/19 antibody by cellulose acetate electrophoresis, pooled, dialysed against distilled water and lyophilised. The yield of protein in one such experiment was 313 mg.

Coupling of YCK5/19 antibody to Sepharose 4B for purification of interferon by immunoadsorption chromatography

YCK5/19 antibody purified as described above was copuled to CNBr-activated Sepharose 4B (Pharmacia) at 10 mg protein/ml of swollen Sepharose as described previously (Secher & Burke, 1980). Greater than 95% coupling was achieved as estimated by measuring the protein concentration in the solution before and after reaction with the Sepharose.

Radiolabelling of YOK5/19 antibody

YOK/19 antibody purified as above was radiolabelled with ^{125}I using chloramine-T as previously described for NK2 (Secher, 1981). A specific activity of about 20 µCi/µg (3Ci/µmole) was obtained.

Ability of YOK5/19 to neutralise Hu-IFN-α

A pool of YOK5/19.8 and YOK5/19.22 sera was test d for its ability to neutralize the activity of Hu-IFN-α in an antiviral assay (Ath rton & Burke, 1975). The results indicate a neutralisation titre

of 0.7 when tested against 2500U of Hu-IFN-α (Ly) (Namalwa) and of 1.8 against 25U Hu-IFN-α . These results indicate that at high antibody concentration YOK5/19 can neutralise the antiviral activity of and of the major components of Namalwa IFN.

Specificity of YOK5/19 and NK2 anti-interferons using various Hu-IFN- preparations

In the selection and cloning of YOK5/19 the assays used NK2 coated plastic wells which showed that the antigenic site recognised by YOK5/19 was different from that recognised by NK2, but that at least some Hu-IFN-α molecules contained both antigenic sites and that both monoclonal antibodies could bind simultaneously. The specificity of the two antibodies was further investigated using sheep anti-interferon coated wells and comparing the cpm bound when different types of Hu-IFN-α were used. The panel of IFNs consisted of crude leucocyte IFN (Hu-IFN-α(Le), the effluent when crude leucocyte IFN was passed through an NK2-Sepharose column and thus depleted of NK2 recognised interferons ("NK2 effluent"), a cloned Hu-IFN-α (D sub-type) and cloned Hu-IFNsα-A, -B, -C, -D, -F, -I, J, K. The cpm bound when no IFN was added to the assay defined the non-specific binding (usually 200-400 cpm) that was subtracted from the other values.

Tables 5 and 6 show the results of different such experiments, and indicate the reproducibility of the assay.

The results clearly show that whereas NK2 recognises A,B,C,D but not F, YOK5/19 is most active with IFN-D.

TABLE 5:

	IFN						
	A	B	C	D	F	Crude IFN-α	NK2 effluent
Rat antiserum	21	15	22	16	13	25	n.d.
NK2	21	11	13	11	0	10	3
YCK5/19	3	0	10	11	0	4	3
YOK5/19 (1)	1	0	9	37	0	7	7
YCK4.I.C6	3	3	3	3	3	10	10

Numbers indicate cpm bound (x 10-2) after subtraction of background cpm obtained when no IFN was added to the assay. YCK5/19 (1) is a subculture of the original YOK5/19. YOK4.1.C6 is a culture from a different fusion that was lost.

TABLE 6

	IFN											
	A	B	C	D	F	I	J	K	Hu-IFN-α 1	Crude IFN-α	NK2-IFN	NK2 Effluent
NK2	24	12	12	5	1	19	4	19	1	19	26	2
YOK5/19	2	1	14	44	3	11	12	3	15	15	5	15
YOK5/19.31 (1/10 dil^n)	0	0	8	35	1	7	7	0	7	6	nd	6
YOK5/19.31.9 (1/100 dil^n)	4	0	20	61	3	17	14	1	17	15	3	12

YCK5/19.31.9 was also tested in a similar assay on Hu-IFN-A from Roche and the results suggested no recognition of A under the conditions of the assay.

Anti-interferon assay used to measure antibody concentrations

The anti-interferon binding assay described above was also used to measure the relative concentration of YCK5/19 antibody in different samplers. For each sample a series of dilutions was prepared and the cpm bound in the assay using a suitable IFN (e.g. IFN-α1 or crude IFN) measured for each dilution. The results of such an experiment are shown in Figs. 1, from which it can be seen that the titre (dilution at which half maximal cpm bound) is between 1/104 and 1/105, about 100 fold more concentrated than in the supernatant of YCK5/19 cells before cloning. A control experiment was performed with no interferon-α present to obtain a background cpm bound value.

Immunoglobulin class of YCK5/19.31.9 antibody

YCK5/19.31.9 cells were grown in 14C-lysine containing medium and the radioactive supernatant subjected to SDS-polyacrylamide gel electrophoresis and autoradiography as described (Galfre & Milstein, 1981). The autoradiography clearly showed the existance of a single (γ) heavy chain and a single light chain, supporting the monoclonal nature of YOK5/19.31.9 and establishing the antibody as an IgG.

A similar conclusion was reached from SDS-polyacrylamide gel electrophoresis of 125I-YOK5/19 purified antibody labelled as described above. The only bands visible in the autoradiograph had the mobility of heavy (γ) and light chains.

Immunoradiometric assays with YOK5/19

Various analogues of the immunoradiometric assay (IRMA) previously described for measuring IFN-α

(Secher, 1981) were constructed and tested. These involved the use of ^{124}I-labelled YOK5/19 antibody prepared as described above or plastic beads (or other solid support) coated with YOK5/19 antibody. This was done as follows: YOK5/19 antibody purified by ammonium sulphate precipitation and ion exchange chromatography as described above, was diluted to 50 µg/ml in PBS, 0.05% NaN_3, 5 mM EDTA and 200 polystyrene beads immersed in this solution for 16h at 4°C. The beads were washed and stored in PBS, 0.1% NaN_3, 0.5% BSA. In an IRMA with YOK5/19-coated beads and radiolabelled YOK5/19 as tracer there was no significant binding of radioactivity at IFN concentrations of up to 10^5U/ml. This indicates the presence of only a single antigenic site for YOK5/19 per molecule of interferon. A similar observation was made for NK2 and is in accord with the monomeric nature of IFN-α molecules.

TABLE 7:

IRMAs for IFN-α using monoclonal antibodies

No.	Solid phase antibody	Radiolabelled antibody	Assay possible
1	Sheep anti-IFN-α	NK2	yes
2	Sheep anti-IFN-α	YOK5/19	yes
3	YOK5	NK2	yes
4	YOK5	YOK5/19	no
5	NK2	NK2	no
6	NK2	YOK5/19	yes

Table 7 lists those combinations of monoclonal antibodies that were tested as possible IRMAs for Hu-IFN-α. A "reverse IRMA" using sheep anti IFN-α in solution has been described (Hawkins & Secher, 1983).

Some of the assays suffered from the same problem of inhibition due to competition for the solid phase by other IFN-α species not recognised by the radiolabelled antibody. This was apparent in those assays which had the polyclonal sheep antibody on the bead (Nos. 1, 2 of Table 7) and to a lesser extent in assay No.6. The combination of YCK5/19 on the solid support with radiolabelled NK2 as tracer showed no sign of this inhibition and gave, at 50,000 U/ml, 3900 cpm bound with 65000 cpm input. Only those IFN-α species that contain both NK2 and YCK5/19 antigenic determinants can be recognised by an assay that uses the two monoclonals and to investigate this specificity further a panel of IFN's was again used (all at approx. 5000 U/ml). The results of this comparison are shown in Figure 2. (A, B, C, D, F, I, J, K, are samples of individual sub types of human interferon-α , Cr is a crude sample of interferon, Ef is the effluent from an NK2 immuno purification column. NK is interferon purified on an NK2 immunopurification column, α1 is a sample of α1 human interferon and b/g is background i.e. control). In Figure 2a the assay involved solid phase NK2 antibody and radiolabelled YOK5/19 antibody. In Figure 2b the assay involved solid phase YOK5/19 antibody and radiolabelled NK2 antibody IFN-α-B, -F, and α1 all seem to be unrecognised in the combined assay. The other species tested are all recognised in at least one of the assays. The different relative sentitivities are probably due to differences in the avidity of the two monoclonal

antibodies to the various species and the fact that the solid phase antibody is at an effective concentration far higher than that of the radiolabelled antibody.

The importance of antibody concentration (and avidity) was demonstrated in a standard anti-interferon assay as described above, in which the anti-interferon was purified YOK5/19 IgG at either 10 μg/ml or 1 μg/ml. For IFN-α-D the cpm bound decreased in one assay from about 1400 at 10 μg/ml to 11000 at 1 μg/ml, but for IFN-α-B the decrease was from about 1500 cpm to around 0 cpm above background. Hu-IFN-α-A, -C, -D were tested over a range of concentrations. The results Figure 3 suggested that differences in relative sensitivities in the two assays could not be the result of imprecise estimates of the IFN concentrations. (Figure 3a 10 μg/ml; Figure 3b 1μg/ml).

A similar analysis of the antigenic specificities of the assays that use a single labelled monoclonal antibody together with a polyclonal solid phase antibody is shown in Figure 4. In Figure 4 the solid phase antibody is sheep anti interferon-α and in Figure 4a the radiolabelled antibody is YOK5/19 antibody and in Figure 4b the radiolabelled antibody is NK2 antibody.

Use of YOK5/19-Sepharose 4B columns for IFN-α-purification

IgG purified from serum and ascites fluid of rats carrying YOK5/19 tumours and coupled to Sepharose 4B as described above has been used in the immunopurification of Hu-IFN-α from leukocytes and from E. coli producing Hu-IFN-α1.

In a pilot experiment a small column of YOK5/19-Sepharose 4B (0.6 ml) was used according to the same protocol as that developed for NK2-S pharose

(Secher & Burke, 1980) to purify IFN-α from 45 mls of crude leukocyte IFN. About 2.2 x 106U IFN- was loaded and about 46% of this IFN-α (as judged by IRMA) was eluted at pH2 and recovered in a single fraction. Trichloracetic acid precipitation of the protein contained in fraction and analysis by SDS-PAGE revealed a single major band of MW 15,000-20,000 and a second much weaker band of slightly higher MW. This result suggests that the IFN may be pure after a single passage through the YOK5/19 Sepharose column.

The specificity data obtained with the anti-interferon assay (see above) suggest that tye YOK5/19 antibody recognises interferon in NK2-column effluent and that YOK5-Sepharose and NK2-Sepharose might be complementary in their purification of IFN from crude IFN mixtures. This was confirmed by the sequential use of NK2-Sepharose and YOK5/19-Sepharose. When crude IFN-α (Le) was passed through an NK2-Sepharose column about 50-60% of the activity estimated by antiviral assay was removed (and could be recovered by elution of the column at pH2). Most of the residual IFN activity was removed by passage through a YOK5-Sepharose column. The antiviral assay suggested that only about 10% of the IFN was not retained by the combination of NK2- and YOK5 Sepharose.

In another experiment crude IFN that had been depleted of NK2 recognised interferon (1400 ml) was passed through a 1 ml column of YOK5-Sepharose. The YOK5-IFN was eluted with 0.1 M citric acid and analysed by SDS-PAGE stained with silver (Wray et al., 1981). The acidic fractions contained most of the IFN activity and a single major band that had the mobility of a marker of Hu-IFN-α1 run on the same gel.

As a further example of the ability of YOK5-Sepharose to purify IFN-α species that cannot be purified by NK2-Sepharose, a crude bacterial lysate (70 ml) containing a recombinant human α1 gene product was passed through a 4.4 ml column of YOK5-Sepharose. (This IFN α1 was not recognised by NK2, either in the IRMA, or by the NK2-Sepharose). The column was washed with PBS and then with 0.1 M ammonium acetate. The bound IFN-α1 was eluted with a volatile pH2 buffer (4.5% (v/v) HCOOH, 0.01 M ammonium acetate) and fractions assayed in an IRMA using sheep anti-interferon on the solid phase and radiolabelled YOK5/19.

The results showed that about 65% of the IFN present in the load was recovered in 2 x 4 ml fractions. The purity of this IFN was shown by SDS-PAGE and silver staining which revealed a single major band of MW about 19000. The purity and authenticity of the purified α1 was confirmed by lyophilisation of the most active fraction and transferring the protein to a Beckman 8903 sequencer for N-terminal amino acid sequence determination. The sequence was in perfect agreement for over 20 residues with that predicted from the DNA sequence of the α1 gene.

Note on nomenclature

In this specification the antibody produced by cells of clone YOK5/19.31.9 (originally called YOK5/19(3)(Ag)(3.80).31.9) or of the similar clones YOK5/19.31, YOK5/19.22, YOK5/19.8, is referred to as YOK5/19 antibody. The abbreviation to YOK5 is analogous to the NK2 abbreviation for NK2/13.35.6 (Secher & Burke, 1980). Interferon purified on YOK5-Sepharose is referred to as "YOK5-IFN", also following an accepted convention for NK2.

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CLAIMS

1. A monoclonal antibody to human interferon-α characterised in that the monoclonal antibody has a greater binding efficiency to D sub-type human interferon-α than to A sub-type human interferon-α.

2. A monoclonal antibody according to claim 1 wherein the monoclonal antibody has a greater binding efficiency to D sub-type human interferon-α than to any of the other sub-types of human interferon-α.

3. A composition comprising, in combination, a monoclonal antibody according to claim 1 or 2 and a monoclonal antibody of complementary binding efficiency.

4. A composition according to claim 3 wherein the monoclonal antibody of complementary binding efficiency is derived from the NK2 cell line.

5. A process for the immunopurification of a sample containing human interferon-α wherein either a monoclonal antibody according to claim 1 or 2 or a composition according to claim 3 or 4 is immobilised upon a solid support to form an immunopurification medium and the sample containing human inteerferon- is contacted with the medium.

6. A process for the immunopurification of a sample containing human inteferon-α wherein the sample is passed sequentially, in either order, through an immunopurification column comprising, immobilised, a monoclonal antibody according to claim 1 or 2 and an immunopurification column comprising, immobilised, a monoclonal antibody of complementary binding efficiency.

7. A process according to claim 5 or 6 wherein the antibody of complementary binding efficiency is derived form the NK2 cell line.

8. An immunoassay for human interferon-α comprising the use of an antibody according to claim 1 or 2.

9. An immunoassay according to claim 8 in which are used a sample to be assayed for human interferon-α

a first antibody to human inteferon-α

the first antibody being bound to a solid phase support and

a second antibody to human interferon-α the second antibody having a label attached thereto

wherein one of the first and second antibodies is a monoclonal antibody according to claim 1 or 2 and the other antibody is either a polyclonal antibody to human interferon-α or a monoclonal antibody of complementary binding efficiency the assay comprising the steps of

placing the sample, the first antibody and the second antibody in contact, in any order or combination, and

measuring the amount of second antibody bound to the solid phase through human interferon-α and the first antibody.

10. An immunoassay for a first antibody to human interferon-α in which is used

a sample to be assayed for the first antibody to human interferon-α,

a solid phase support having bound thereto a second antibody to human interferon-α, the second antibody being bound to human interferon-α

a third antibody, the third antibodfy having a label attached thereto and being capable of binding to the first antibody

the assay comprising the steps of

placing the solid phase support in contact with the sample, thereby allowing first antibody to bind to the human interferon-α attached to the solid phase through the second antibody, placing the solid phase support in contact with the third antibody thereby allowing the third antibody to bind to any first

antibody which is attached to the solid phase through the human interferon-α and the second antibody, and measuring the amount of third antibody associated with the solid phase.

11. A process for the production of a hybridoma cell line capable of producing a monoclonal antibody according to claim 1 or 2 comprising the steps of immunizing an animal with human interferon-α, allowing the immune system of the animal to generate lymphocytes to human interferon-α, preparing a sample of spleen cells taken from the animal, fusing the spleen cells with myeloma cells to form a colony of hybridoma cells, and screening the colony of hybridoma cells for cells screting monoclonal antibody according to claim 1 or 2, wherein the screening step employs an assay according to claim 10, the third antibody having been passed through an immunopurification column, comprising immobilised antibody of a complementary binding efficiency, prior to use in the assay.

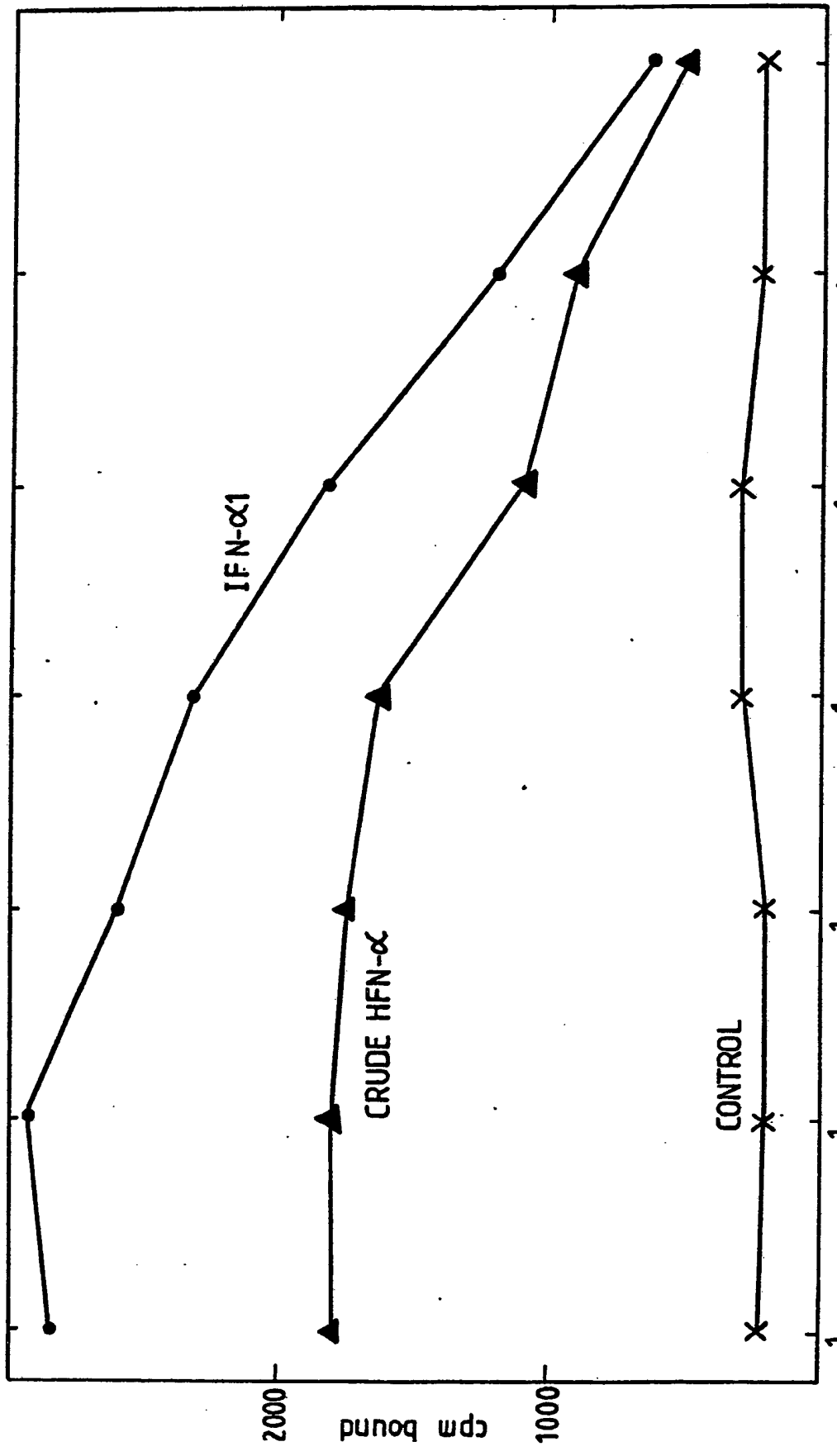

IFN-α1
CRUDE HFN-α
CONTROL
cpm bound
2000
1000
1/1
1/10
1/10²
1/10³
1/10⁴
1/10⁵
1/10⁶
Dilution of culture supernatant

Fig.1.

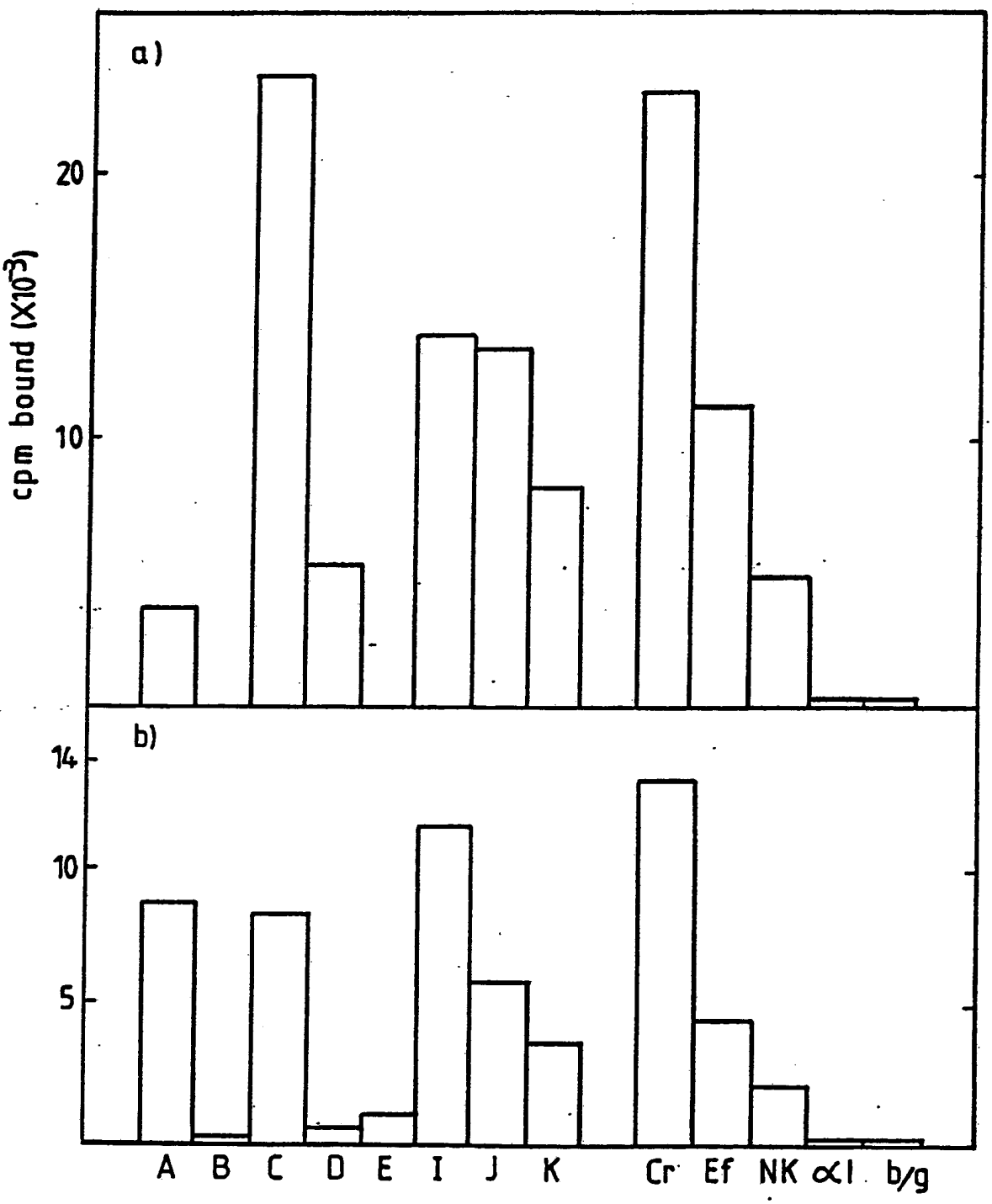
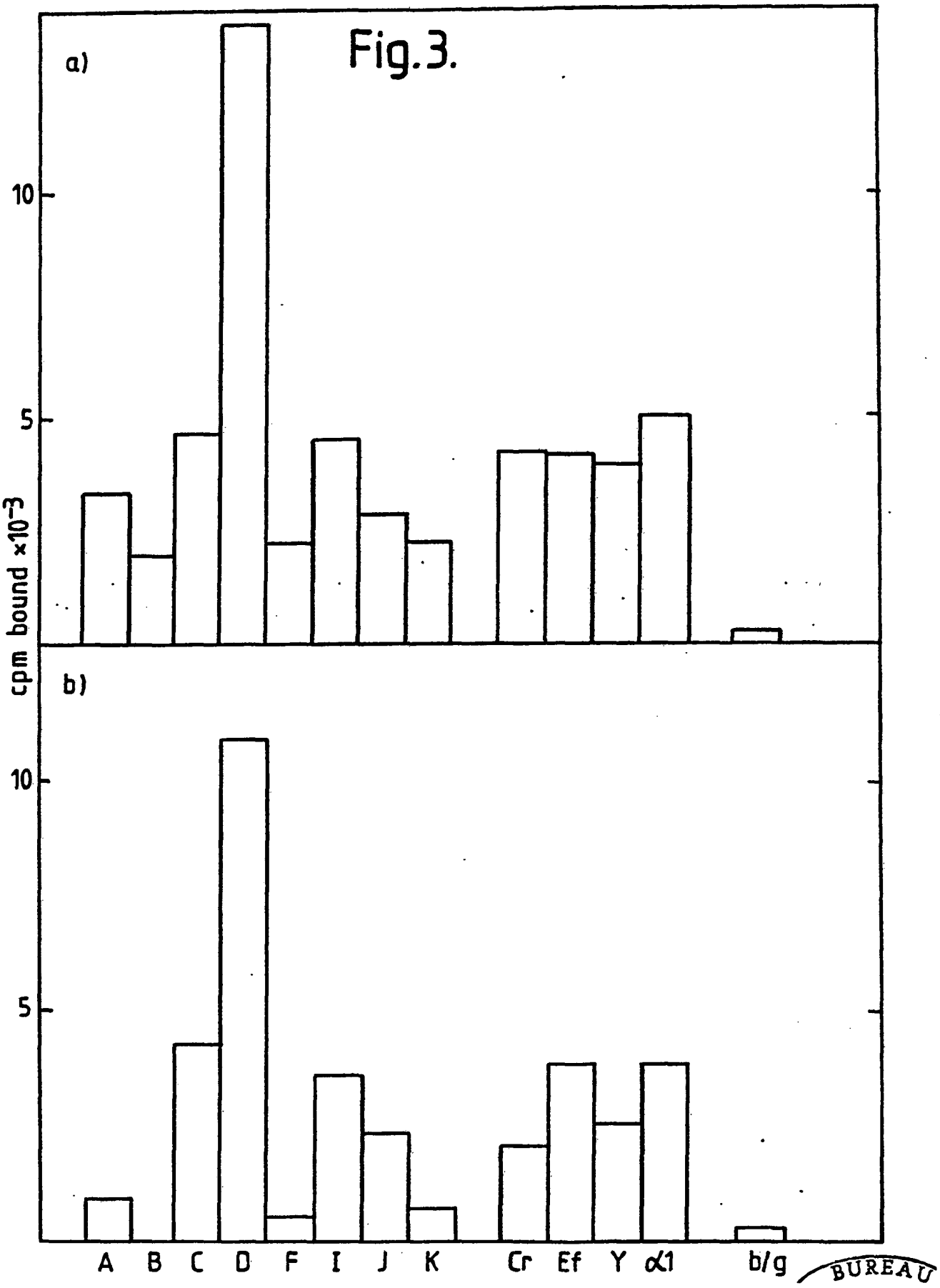


Fig.2.

Fig.3.



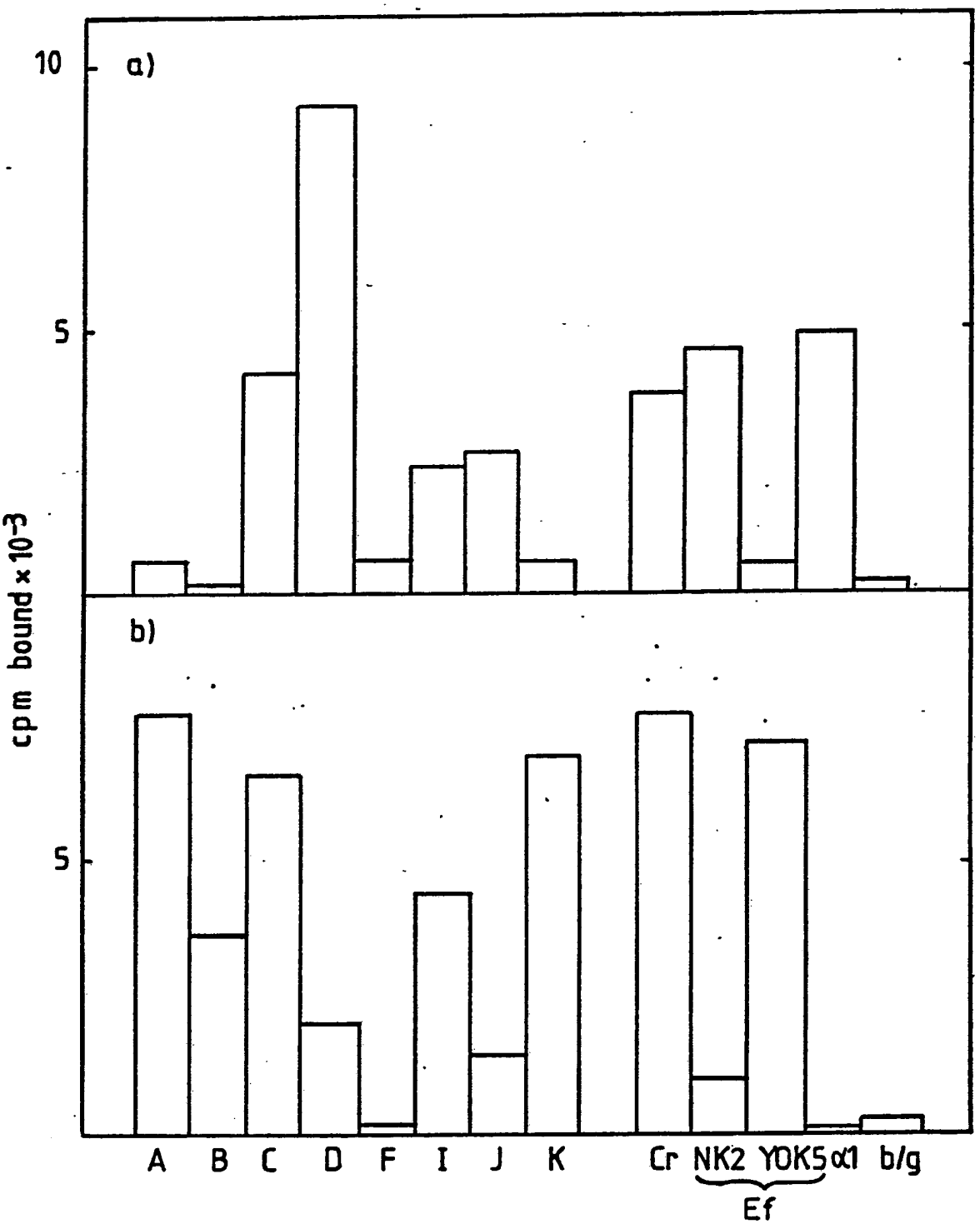


Fig.4.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 84/00031

I. CLASSIFICATI N F SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[3]: C 12 P 21/00; C 12 N 15/00; C 07 C 103/52; G 01 N 33/54

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC[3]	C 12 P; C 12 N; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [5]

III. D CUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	Nature, vol. 294, no. 5838, 19 November 1981 (Chesham, Bucks, GB) H. Arnheiter et al.: "Physicochemical and antigenic properties of synthetic fragments of human leukocyte interferon", pages 278-280, see page 279, Table 1 and page 280, right-hand column, lines 9-15	1,2,5
Y		3-11
Y	FR, A, 2500754 (HOFFMAN-LA ROCHE) 3 September 1982 see claims 1,2,10-13; page 2, line 26 - page 3, line 5; examples 1 and 2	3,5,6,10,11
Y	WO, A, 82/01773 (CELL-TECH LTD.) 27 May 1982 see the claims and page 4, lines 24-32	4,7-9
A	WO, A, 81/02899 (D.S. SECHER et al.) 15 October 1981 see the claims (cited in the application)	1-11

* Special categories of cited documents: [15]

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"&" document member of the same patent family

IV. CERTIFI ATION

Date of the Actual Completion of the International Search [3]	Date of Mailing of this International Search Report [3]
10th May 1984	19 JUIN 1984
International Searching Authority [1]	**Signature of Authorized Officer** [20]
EUROPEAN PATENT OFFICE	G.L.M. Kruydenberg

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 84/00031 (SA 6529)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 08/06/84

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
FR-A- 2500754	03/09/82	BE-A-	892296	26/08/82
		DE-A-	3206743	23/09/82
		NL-A-	8200786	16/09/82
		AU-A-	8077282	02/09/82
		JP-A-	57197224	03/12/82
		SE-A-	8201226	24/09/82
		LU-A-	83978	07/06/83
		GB-A-	2111527	06/07/83
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		GB-A-	2083836	31/03/82
		EP-A-	0064063	10/11/82
		AU-A-	7035781	26/10/81
WO-A- 8102899	15/10/81	GB-A-	2083836	31/03/82
		EP-A-	0050129	28/04/82
		AU-A-	7035781	26/10/81
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